PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 7/08, 7/10, 13/00
A61K 39/09, 39/40, C12P 21/08
G01N 33/569

A1

(11) International Publication Number: WO 93/21220

(43) International Publication Date: 28 October 1993 (28.10.93)

(21) International Application Number: PCT/AU93/00131

(22) International Filing Date: 30 March 1993 (30.03.93)

(30) Priority data:
PL 1800 8 April 1992 (08.04.92) AU

(71) Applicant *(for all designated States except US)*: THE COUNCIL OF THE QUEENSLAND INSTITUTE OF MEDICAL RESEARCH [AU/AU]; 300 Herston Road, Brisbane, QLD 4029 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : GOOD, Michael, Francis [AU/AU]; 46 Weemala Street, The Gap, QLD 4061 (AU). PRUKSAKORN, Sumalee [TH/AU]; The Council of the Queensland Institute of Medical Research, 300 Herston Road, Brisbane, QLD 4029 (AU).

(74) Agents: SLATTERY, John, M. et al.; Davies Collison Cave, 1 Little Collins St., Melbourne, VIC 3000 (AU).

(81) Designated States: AU, BR, CA, KR, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: SYNTHETIC PEPTIDES USEFUL IN A VACCINE AGAINST AND IN THE DIAGNOSIS OF STREPTOCOCCAL INFECTION

(57) Abstract

The present invention provides a synthetic peptide comprising at least on B cell epitope from the carboxyterminus of M protein of Group A ß-hemolitic streptococci wherein an antibody reactive to the B cell epitope is only minimally reactive to human heart tissue. The synthetic peptide may also contain a T cell epitope. The present invention further comtemplates a vaccine comprising the synthetic peptide and a method for vaccinating human subjects against streptococcal infection.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

SYNTHETIC PEPTIDES USEFUL IN A VACCINE AGAINST AND IN THE DIAGNOSIS OF STREPTOCOCCAL INFECTION

The present invention relates generally to vaccines and diagnostic reagents suitable for use against streptococcal infection. More particularly, the present invention relates to synthetic peptides based on the M protein of streptococci and carrying one or more B and/or T cell epitopes.

Group A streptococci are responsible for many suppurative infections, but the etiologies of two-non-suppurative inflammatory conditions - rheumatic fever and its complications, and acute glomerulonephritis - are not well understood. Acute rheumatic fever follows a throat infection with certain serotypes of group A β-hemolytic streptococci (Kaplan et al., 1989). This disease, and the recurrences which can follow further streptococcal throat infections, expresses its most serious pathology in the heart. It has been suggested that the disease represents an autoimmune illness initiated as a result of cross-reactivity between the M protein of streptococci and cardiac tissue (Cunningham and Russell, 1983; Cunningham et al., 1986; 1988; Dale and Beachey, 1986; Ayakawa et al. 1988; Fenderson et al., 1989). Immunity to gruop A β-hemolytic streptococci is nevertheless indicated by antibodies to the M protein. However, a subunit vaccine to prevent rheumatic fever based on the M protein has hithertofore not been possible due to the presence of potentially deleterious epitopes, i.e. those cross-reactive with heart tissue and to the fact that the aminoterminal half of the protein is highly variable. There is a need, therefore, to develop a subunit vaccine that contains epitopes which are targets of neutralising antibodies, but which do not contain heart cross-reactive epitopes.

In work leading up to the present invention, the immunogenicity of epitopes located in the highly conserved carboxyterminus of the protein were examined to locate T cell epitopes and/or conserved B cell epitopes.

In accordance with the present invention, peptides have been synthesised carrying epitopes reactive to antibodies to M protein but only minimally reactive, if at all, to human heart tissue. Such peptides will be useful in the development of a vaccine against streptococcal infection and in particular rheumatic fever and in developing a range of diagnostic reagents for streptococcal infection.

Accordingly, one aspect of the present invention provides a synthetic peptide comprising at least one B cell epitope from the carboxyterminus of M protein of Group A β-hemolytic streptococci wherein an antibody reactive to the B cell epitope is only minimally reactive to human heart tissue.

Another aspect of the present invention is directed to a synthetic peptide comprising at least one B cell epitope from the carboxyterminus of M protein of Group A hemolytic streptococci and at least one T cell epitope, wherein an antibody reactive to the B cell epitope is only minimally reactive to human heart tissue.

In one embodiment, the T cell epitope is also from the carboxyterminus of M protein or may be part of an extraneous or fusion molecule with the synthetic peptide. The term "synthetic" extends to recombinant and chemically synthesised molecules as well as biologically pure naturally occurring molecules.

By "minimally reactive" is meant that there is less than 35%, preferably less than 25%, more preferably less than 15%, even more preferably less than 5% and most preferably 0-2% binding of the antibody to heart tissue compared to the B cell epitope or the synthetic peptide.

The term "peptide" is used in a general sense to cover molecules ranging from a few amino acid residues to several hundred amino acid residues. Furthermore, the term "peptide" is considered herein to cover a polypeptide.

The peptides of the present invention are henceforth referred to as "M peptides" for the M protein of streptococci. The M peptides of the present invention do not extend to the full length M protein.

The M peptides of the present invention may comprise an amino acid sequence exactly corresponding to a sequence in a region of the naturally occurring carboxyterminus of M protein or may contain single or multiple amino acid substitutions, deletions and/or additions to the naturally occurring sequence. The carboxyterminus is construed herein in its most general sense and extends from amino acid residues 270 to 492, more particularly 300 to 492 and most particularly 337 to 492 of the type 5 M protein.

The M peptides contemplated herein may be chemically synthesized such as by solid phase peptide synthesis or may be prepared by subjecting the M protein to hydrolysis or other chemically disruptive processes to produce fragments of the molecule. Alternatively, the peptides can be made by in vitro or in vivo recombinant DNA synthesis. In this case, the peptides may need to be synthesised in combination with other proteins and then subsequently isolated by chemical cleavage or alternatively the peptides or polyvalent peptides may be synthesised in multiple repeat units. Furthermore, multiple epitope peptides or polyvalent peptides can also be prepared according to Tam (1988). The selection of a method of producing the subject peptides will depend on factors such as the required type, quantity and purity of the peptides as well as ease of production and convenience.

The use of these M peptides in vivo may first require their chemical modification since the peptides themselves may not have a sufficiently long serum and/or tissue half-life. Chemical modification of the subject peptides may also be important to improve their antigenicity including the ability for certain regions of the peptides to act as B and/or T cell epitopes. Such chemically modified M peptides are referred to herein as "analogues". The term "analogues" extends to any functional chemical or recombinant equivalent

of the M peptides of the present invention characterised by their possession of at least one B cell epitope from the M protein of Group A β-hemolytic streptococci and wherein an antibody reactive to the B cell epitope is only minimally reactive with human heart tissue. The term "analogue" is also used herein to extend to any amino acid derivative of the peptides as described above.

Analogues of M peptides contemplated herein include, but are not limited to, modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptides or their analogues.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6, trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5'-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation via O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic

acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids.

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain

and the N or C terminus.

The present invention, therefore, extends to peptides and polypeptides and amino acid and/or chemical analogues thereof corresponding to one or more regions of the M protein acting as at least one B cell epitope and which may also contain at least one T cell epitope as defined above.

The preferred M peptides of the present invention include residues representing the conserved region of the M protein and in particular amino acid residues 337 to 492 of the type 5 M protein, including any derivatives or analogues thereof as contemplated above as well as multiple repeats and/or combinations from this region. However, all useful peptides from the carboxyterminus or variations thereof as hereinbefore defined, are encompassed in the present invention. In a most preferred embodiment of the present invention, the M peptides are defined by the following amino acid sequences which corresponds to peptide 145 in Table 1:

LRRDLDASREAKKQVEKALE

The present invention further contemplates a method for vaccinating a human subject against streptococci infection comprising administering to said subject a humoral immunity developing effective amount of a peptide comprising at least one B cell epitope from the M protein of Group A hemolytic streptococci and at least one T cell epitope wherein an antibody reactive with the B cell epitope is only minimally reactive with heart tissue for a time and under conditions sufficient for said immunity to develop or at least partially develop. Such a peptide has the same meaning as set forth above and the T cell epitope may or may not be from the M protein carboxyterminus although, in a preferred embodiment, it is located on the M peptide. In an alternative embodiment, the T cell epitope is separate to the M peptide and may, for example, be part of an adjuvant, such as Alum. Generally, this aspect of the present invention can be accomplished by a vaccine composition.

Accordingly, another aspect of the present invention contemplates a vaccine useful in the development of humoral immunity to M protein but minimally cross reactive with heart tissue said vaccine comprising a peptide carrying at least one B cell epitope from the M protein and at least one T cell epitope wherein an antibody reactive with said B cell epitope is only minimally reactive with heart tissue and said vaccine further comprising one or more pharmaceutically acceptable carriers and/or diluents. Such an M peptide is as defined above.

The vaccine may contain a single peptide type or a range of peptides covering different or similar epitopes. In addition, or alternatively, a single polypeptide may be provided with multiple epitopes. The latter type of vaccine is referred to as a polyvalent vaccine.

The formation of vaccines is generally known in the art and reference can conveniently be made to Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pennsylvania, USA.

The present invention, therefore, contemplates a pharmaceutical composition or vaccine comprising a humoral immunity developing effective amount of M peptide or its derivatives, analogues or homologues and/or combinations thereof including other active molecules and one or more pharmaceutically acceptable carriers and/or diluents. The active ingredients of a pharmaceutical composition comprising the M peptide are contemplated herein to exhibit excellent therapeutic activity, for example, in the development of antibodies to M protein of streptococci but said antibodies being only minimally reactive with heart tissue when administered in amount which depends on the particular case. For example, from about 0.5 ug to about 20 mg per kilogram of body weight per day may be administered. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic

situation. The active compound may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (eg using slow release molecules). Depending on the route of administration, the active ingredients which comprise an M peptide may be required to be coated in a material to protect said ingredients from the action of enzymes, acids and other natural conditions which may inactivate said ingredients. For example, the low lipophilicity of the M peptides will allow them to be destroyed in the gastrointestinal tract by enzymes capable of cleaving peptide bonds and in the stomach by acid hydrolysis. In order to administer M peptides by other than parenteral administration, they will be coated by, or administered with, a material to prevent its inactivation. For example, M peptides may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes.

The active compounds may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and

storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as licithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the M peptides are suitably protected as described above, the active, compound may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of

ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 ug and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the

art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 µg to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5 µg to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

Still another aspect of the present invention is directed to antibodies to the M peptides. Such antibodies may be monoclonal or polyclonal and may be selected from naturally occurring antibodies to the M protein or may be specifically raised to the M peptides. In the case of the latter, the peptides

may need first to be associated with a carrier molecule. The antibodies and/or M peptides of the present invention are particularly useful for immunotherapy and vaccination and may also be used as a diagnostic tool for infection or for monitoring the progress of a vaccination or therapeutic regima.

For example, the M peptides can be used to screen for naturally occurring antibodies to M protein. Alternatively, specific antibodies can be used to screen for M protein. Techniques for such assays are well known in the art and include, for example, sandwich assays and ELIZA.

In accordance with this aspect of the present invention, the M peptides are particularly useful in screening for antibodies to M protein and, hence, provide a diagnostic protocol for detecting streptococcal infection. Alternatively, biological samples, such as blood serum, sputum, tissue and tissue extracts can be directly screened for M protein using antibodies raised to the M peptides.

Accordingly, there is provided a method for the diagnosis of streptococcal infection in a subject comprising contacting a biological sample from said subject with an antibody binding effective amount of an M peptide for a time and under conditions sufficient for an antibody-M peptide complex to form, and then detecting said complex.

The presence of M protein antibodies in a patient's blood serum, tissue, tissue extract or other bodily fluid, can be detected using a wide range of immunoassay techniques such as those described in US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. This includes both single-site and two-site, or "sandwich", assays of the non-competitive types, as well as in the traditional competitive binding assays. Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an M peptide is immobilised onto a solid substrate to form a

first complex and the sample to be tested for M protein antibody brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an M-peptide-antibody secondary complex. An anti-immunoglobulin antibody, labelled with a reporter molecule capable of producing a detectable signal, is then added and incubated, allowing time sufficient for the formation of a tertiary complex of M peptide-antibody-labelled antibody. Any unreacted material is washed away, and the presence of the first antibody is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations of the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody, or a reverse assay in which the labelled antibody and sample to be tested are first combined, incubated and then added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, and the possibility of minor variations will be readily apparent. A similar approach is adopted to detect M protein. The antibodies used above may be monoclonal or polyclonal.

The solid substrate is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs or microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing the molecule to the insoluble carrier.

By "reporter molecule", as used in the present specification, is meant a molecule which, by its chemical nature, profices an analytical identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecule

in this type of assay re either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes). In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognised, however, a wide variety of different conjugation techniques exist which are readily available to one skilled in the art. Commonly used enzymes include horseradish peroxidase, glucose oxidase, B-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production,upon hydrolysis by the corresponding enzyme, of a detectable colour change. It is also possible to employ fluorogenic substrates, which yield a fluorescent product.

Alternatively, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state of excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining ternary complex is then exposed to the light of the appropriate wavelength, the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed. It will be readily apparent to the skilled technician how to vary the procedure to suit the required purpose. It will also be apparent that the foregoing can be used to label M peptides and to use same directly in the detection of M protein antibodies.

The present invention is further described by reference to the following non-limiting Figures and Examples.

In the Figures:

Figure 1 is a graphical representation showing the proliferative response of lymph node cells from B10(A,E), B10.D2(B,F), B10.BR(C,G) and B10.S(7R)(D,H) mice after immunization with pools of 6 peptides (A,B,C,D) or individual peptides (E,F,G,H). The peptide pools were as follows:145-150, 151-156, 157-162. In experiments A-D, the different bars refer to separate experiments as listed below each section of the figure; in experiments E-H, the different bars refer to the different antigens used *in vitro*, as listed below Fig. 1H.

Figure 2 is a photographic representation of an immunoblot of porcine heart myosin (lane 1), HCl-extract M protein (lane 2) and human heart tissue protein (lane 3). Membranes were reacted against anti-porcine heart myosin antisera (A); anti-M-5 cell extract antisera (B); normal mouse serum (C); B10.BR anti-peptide 145 antisera (D); B10.BR anti-peptide 146 antisera (E); B10.D2 anti-peptide 149 sera (F); B10 anti-peptide 150 antisera (G); B10.BR antipeptide 151 antisera (H) and B10.S(7R) anti-peptide 151 antisera (1). Relative molecular masses ($x10^{-3}$) are listed at the left of the Figure.

Figure 3 is a graphical representation showing mean positive optical density readings of ELISA assays of serum antibodies from (A) caucasian and (B) Aboriginal subjects against individual peptides. See Example 2 for abbreviations.

EXAMPLE 1

MAPPING B AND T CELL EPITOPES

1. Materials and Methods

Peptides.

Fifteen overlapping peptides representing the conserved region of the M-5 protein (Miller *et al*, 1988), 3 peptides representing the variable amino-

terminal regions of the M-5, M-6 and M-24 proteins (Miller *et al*, 1988; Hollingshead *et al*, 1986; Mouw *et al*, 1988), two peptides representing human heart myosin (Kurabayashi *et al*, 1988) and one peptide representing human skeletal muscle myosin (Saez *et al*, 1986) were synthesized by the "tea-bag" method (Houghten, 1985). Purity was checked by HPLC. Their sequences are listed in Table 1. Peptides were dissolved in PBS at a concentration of 10 mg/ml and kept at -20C until used.

Streptococcal M-5 protein extract (Rotta, et al. 1980).

M-5 group A streptococci were cultured in 80 ml Todd-Hewitt broth with 20% v/v fetal calf serum, incubated at 37C for 16 hours. Cells were centrifuged and washed (X3) with sterile PBS pH 7.4 and resuspended in HCl pH 2. The suspension was then boiled for 10 minutes in water bath. The pH was adjusted to 7.0 by NaOH and centrifuged at 3000 rpm for 10 minutes, the supernatant was isolated and checked for sterility by dropping on blood agar plate.

Heart tissue proteins.

Porcine heart myosin and tropomyosin were purchased from Sigma Chemical Co. Human heart atrial tissue was derived from surgical specimens and were cut into small pieces and macerated in an homogenizer with cold PBS pH 7.4, centrifuged, supernatant were kept at -20C until used. Pellets were suspended in SDS-PAGE sample buffer and boiled for 5 minutes, centrifuged, and supernatants were kept at 4C for running SDS-PAGE. Human heart pericardial tissue was derived from surgical specimens and macerated in cold PBS pH 7.4 and centrifuged at 3000 rpm; the supernatant was isolated and kept at -20C until used.

Lymph node cell proliferation assays.

Peptides were administered subcutaneously at the tail base (either in pools of 6 or individually) to H-2 congenic mice on the B10 background [B10($H2^b$); B10.D2($H2^d$); B10. BR($H\text{-}2^k$); B10.S(7R)($H\text{-}2^s$)], with each mouse receiving 30 µg of peptide emulsified in complete Freund's adjuvant (CFA) (Difco). Ten

days later, lymph node cells were then plated in quardruplicate at $4x10^5$ per well in flat-bottomed microtitre plates in MEM medium containing 2% v/v heat inactivated normal mouse serum, $5xl0^{-5}$ M 2-mercaptoethanol, 20 µg gentamicin and antigen (at various concentrations). The plates were placed in a humidified 5% v/v CO_2/air environment at 37C for 4 days and then each well was pulsed with 1 µCi ^{3}[H]thymidine for 16 hrs. Incorporation of label was measured by liquid scintillation spectroscopy using a β-plate counter (LKB). A "Stimulation Index" (SI) was defined as: {cpm (in the presence of antigen)}/{cpm (in the absence of antigen)}.

Antibody Production.

Mice were immunized subcutaneously with 30 µg of peptide emulsified in CFA. Mice were given subsequent booster injections at intervals of 10 days with 30 µg peptide dissolved in PBS for 3 boosters. Prior to boost, mice were bled via the tail artery and sera prepared and kept at -20C until used.

ELISA for determining murine antibodies.

Antigen was diluted to 5 µg/ml in carbonate-bicarbonate buffer (pH9.6) and coated onto polyvinyl chloride microplates (Flow Laboratory) in a volume of 100 µl per well overnight at room temperature. The antigen was then removed and the wells blocked with 200 µl of 2% w/v bovine serum albumin (BSA) for 1 hr at 37C. Plates were then washed 5 times with PBS-Tween 20 buffer.

Serum dilutions were prepared in 0.5% w/v BSA PBS-Tween 20 (BSA-PBS-T) buffer and incubated for 1 hr at 37C. The plates were then washed and peroxidase-conjugated goat-anti-mouse IgG (Tago Inc.) was then added at a dilution of 1:4000 in BSA-PBS-T for 1 hr at 37C. After further washing, ABTS substrate in citric acid and H_2O_2 was applied and incubated for 30 mins, the optical density was measured at 405 nm in an ELISA reader (Multiscan, Flow Laboratories). The lowest dilution that gave an OD of greater than 3SD above the mean OD of control wells (containing normal mouse serum at the same dilution) was defined as the titre.

ELISA for determining human antibodies.

One hundred µl of peptide at 5 µg/ml was coated on polypropylene plate (Linbro, Flow) in carbonate coating buffer pH 9.6 (Na_3CO_3 1.9292 g, $NaHCO_3$, 3.8052 g, distilled water 1L) and left at room temperature overnight or at 4 °C for long term storage. Antigen was discarded and wells blocked with 4% skimmed milk in PBS pH 7.4 and 0.05% Tween 20, incubated at 37 °C for 1 hr and washed 5 times. One hundred µl of serum diluted 1:40 was added and incubated at 37 °C for 1 hr. After 5 washes, one hundred µL of goat anti-human IgG at 1:3000 dilution was added and incubated at 37 °C for 1 hr, then washed 5 times with PBS pH 7.5. ABTS (2,2'-AZINO-bis-ETHYLBENZTHIAZOLINE-6-SULFONIC ACID) (Sigma) with H_2O_2 was added and incubated for 30 min. The reactions were measured by spectrophotometer at 405 nm. We are confident that the peptides bound to the wells since all peptides were recognized by sera from at least one individual (titer > 50; OD > 0.5). Furthermore murine antisera raised against these many of these peptides bound the peptides in the wells at titres > 6,400 (14).

SDS-PAGE and immunoblot.

Slab gel electrophoresis (Laemmli, 1970) with modification was performed in 7% w/v acrylamide gel using the Biorad minigel apparatus. Fifteen microliter of porcine heart myosin, human heart tissue protein and M-5 protein in sample buffer (0.05 M Tris-HCl, 0.002 M EDTA, 10% v/v glycerol, 1% w/v SDS, 1% v/v mercaptoethanol and bromphenol blue) were applied to the gel and run for 45-60 minutes until the indicator bromphenol blue reached the bottom of the gel, at which time the gel was removed and blotted for 1 hr onto a nitrocellulose membrane (0.45 µm pore size) in transfer buffer. The blot was blocked with 3% w/v skim milk with 0.05% v/v Tween 20 overnight at 4C. The membrane was then added to 1:100 dilution of mouse serum containing putative antibodies for 1 hr at 37C, then 1:1000 dilution of peroxidase conjugated goat-anti-mouse IgG in 3% w/v skim milk without Tween 20 for 1 hr at 37C. Membranes were washed 5 times with PBS between steps. The

antigen bands were observed after the addition of substrate (4-chloro-1-naphthol in methanol and H_2O_2) for 10 min. The reaction was stopped by washing with distilled water.

Bactericidal assay.

M-5 group A streptococci were cultured overnight in 1 ml Todd-Hewitt broth at 37C, centrifuged and resuspended in 10 ml skim milk broth and stored at -70C until used. Before the assay, the bacteria were thawed and diluted to 10^{-5} dilution in sterile normal saline. Fifty µl of the bacterial dilution was then mixed with 50 µl of mouse serum and 400 µl of normal heparinized human blood was added. The mixture was then incubated with end over end rotation at 37C for 3 hrs, and 50 µl of the mixture was diluted and spread onto blood agar plates and colonies counted after 24-48 hrs of incubation. The percentage of bacteria killed was determined by counting the number of colonies growing following incubation with test sera and comparing this with the number of colonies growing following incubation in control sera.

2. RESULTS

Four different strains of mice which differ only at their MHC loci (H-2) were immunized at the tail base with groups of pooled peptides (6 peptides per pool) which overlapped the carboxylterminal conserved half of the M protein and the aminoterminal variants (Table 1, peptides 145-162). Draining lymph node cells were then challenged *in vitro* with individual peptides. This protocol is designed to optimize the screening procedure for T cell epitopes. Six mice were used for each immunization and the experiments were each performed 2 or 3 times for each strain/pool combination (Figure 1 A,B,C,D). A number of possible T cell epitopes were thus identified for each strain. These peptides were then used individually to immunize mice of the appropriate strain. Lymph node cells were then challenged with the individual peptides, Purified Protein Derivative of *Mycobacterium bovis* (PPD) (as a positive control), tropomyosin and an extract of human pericardial heart tissue (see Example 1) (Figure 1

E,F,G,H). There were generally good responses to the specific peptides and to PPD, although not all peptides were able to reproducibly stimulate T cell responses, reflecting differences in the degrees of immunodominance of the various peptides (Gammon *et al*, 1987; Good *et al*, 1990). However, there were only minimal responses to tropomyosin and heart tissue extract. Conversely, all strains were immunized with an extract of human heart tissue and draining lymph node cells were challenged with heart tissue and the various peptides. While there was a very vigorous response to heart tissue extract (SI range: 50-123), there was no proliferation to the M protein peptides (SI all <2). To further determine the extent of any T cell cross-reactivity between the M protein and heart or muscle myosin peptides, mice were immunized with peptides from the M protein and draining lymph node cells stimulated *in vitro* with porcine heart myosin as well as peptides representing human heart myosin and human skeletal muscle myosin (chosen because of their sequence homology with M protein as shown in Table 2). The results are shown in Table 3. Although peptide 146-specific T cells from B10.BR mice responded to porcine myosin, the response (SI: 3.57) was much less than the response to peptide 146 (SI: 38.95). Thus, the results are consistent with there being no response or minimal response of M peptide-specific T cells with myosin antigens.

To determine whether the proliferative T cell epitopes represented "helper" T cell epitopes, mice were immunized subcutaneously with the individual peptides emulsified in CFA, boosted with aqueous peptide (X3), and serum analysed for the presence of peptide-specific antibodies. Many peptides stimulated antibody production indicating that many of the proliferative T cell epitopes were also helper epitopes (Table 4).

In order to determine whether T and B cells recognised the same regions of these 20-mer peptides, mice were immunized with individual peptides, and then T cells and antibodies tested for reactivity to overlapping peptides (overlap of 10 amino acids). Table 5 lists the various stimulation indices and

ELISA titres for the immunodominant epitopes. In most cases, T and B cell epitopes appeared to map to similar regions of the peptides. With respect to peptide 149 antibodies from B10.D2 mice reacted well with both peptides 148 and 149, whereas peptide 149-specific T cells respond well to peptide 149, but not at all to peptide 148.

Cross-reactivity of serum antibodies to porcine heart myosin was also determined. In the first set of experiments, different mice were immunized with various peptides and the results of pooled or individual sera are given in Table 6 for porcine myosin-reactive antisera. Following this, a separate set of experiments was performed (Table 7) where further mice were immunized and boosted with either peptides 149 or 151- the peptides most strongly associated with induction of anti-myosin responses in Table 6. As shown, (Tables 6 and 7), even though these mice are inbred H-2 congenic animals, differences were noted between individual animals in their ability to generate anti-myosin antibodies, although all animals in Table 7 produced anti-peptide antibodies. When positive sera were confirmed with immunoblot, antibodies to peptides 145, 146, 149, 150, and 151 reacted with porcine heart myosin (Fig. 2 D-I). There was also a moderate reaction with anti-peptide 146 (B10.BR), 150 (B10), and 151 (B10.BR) antisera to human atrial tissue (Fig. 2 E,G,H). Antibody to porcine heart myosin reacted with acid-extracted M-5 protein and strongly with human heart tissue (Figure 2A), and antibody to acid extract of M-5 protein also reacted with porcine heart myosin and human atrial tissue proteins (Figure 2B).

Antibodies to the conserved peptides as well as antibodies to the type-specific aminoterminal B cell epitopes were tested for their ability to kill serotype 5 group A streptococci in a bactericidal assay (Table 8). As previously described, antibodies to peptide 160 (aminoterminal epitope from type M-5) were associated with significant killing (Beachey *et al*, 1988); however, the inventors were able to reproducibly show that antibodies to peptides 145.

EXAMPLE 2

MAPPING HUMAN B AND T CELL EPITOPES

1. Materials and Methods

The following abbreviations are used below and in Figure 3.

ARFA, Aborigines with acute rheumatic fever (RF) (n=6);

RHDA, Aborigines with rheumatic heart disease as a result of prior RF (N=23);

PARFA, Aborigines having a past history of RF, but not having any present clinical evidence of rheumatic heart disease (N=6);

CHOREAA, Aborigines with previous rheumatic chorea without rheumatic heart disease (N=1);

CRHDA, Aborigines with both past rheumatic chorea and rheumatic heart disease (N=3);

HEARTA, Aborigines having other (non-rheumatic) forms of heart disease (N=15);

NORMALA, Aborigines having no history of heart disease (N=32);

PARFC, Caucasians having a past history of RF, but not having any present clinical evidence of heart disease (N=27);

HEARTC, Caucasians having other (non-rheumatic) forms of heart disease (N=23);

NORMALC, Caucasians having no history of heart disease (N=28).

Patients were assessed by a specialist physician. Those with rheumatoid heart disease (RHD) had unequivocal clinical evidence of established significant mitral +/- aortic valve damage, confirmed by echocardiogram in the majority of cases. Those with primary or recurrent rheumatic fever (RF) fulfilled the revised Jones criteria for diagnosis of RF. (Special Committee Report, Jones criteria revised for guidance in the diagnosis of Rheumatic Fever, Circulation publication 69: 203A-208A, 1984). A small number of patients also manifested classical rheumatic chorea. All controls were examined and found to have no clinical evidence of RHD in addition to having no past history of RF.

Antigens.

Overlapping synthetic peptides spanning the conserved region of the M 5 protein were produced as described in Example 1. Human heart pericardial tissue was derived from surgical specimens as described in Example 1.

Lymphocyte proliferation assays.

Twenty five millilitres of blood were drawn. Peripheral blood mononuclear cells (PBM) were prepared by layering blood diluted in an equal volume of MEM over Ficoll-paque, centrifuging at 400 g for 25 min, and collecting the buoyant cells. Cells were washed, counted and added to round-bottomed microtiter wells at a concentration of 200,000 cells per well in MEM medium containing 10% v/v normal human serum, and gentamicin (20 µg/ml). Some wells contained peptide at optimal concentration (30 µg/well), whereas other wells without peptide were used as control wells. After 6 days, wells were pulsed with 0.25 µCi of ^{3}H-methyl thymidine, and 16 hrs later, wells were harvested onto filter paper and thymidine incorporation estimated by liquid scintillation spectroscopy using an LKB beta-plate system. The mean counts per minute (CPM) of wells with peptide was divided by the mean CPM of wells without peptide to give a stimulation index (SI).

Serum isolation.

Three millilitres of blood were put in a sterile tube and let stand at room temperature for 3-4 hours. The serum was isolated by centrifugation and stored at -20 °C.

T cell line generation.

The method is generally as described in Good et al (1987). The peripheral blood lymphocytes from a donor (GL) were stimulated with 150 µg/ml of HCl extracted M protein *in vitro* and incubated at 37 °C in 5% v/v CO_2 atmosphere for 6 days. Aliquols of 30 units/ml of r-IL-2 was then added for 7-10 days and the cells were stimulated again with the same amount of peptide with 4 x 10^6 autologous PBMC without IL-2. After further expansion with IL-2 for 10-14

days, the lines were assayed.

Phenotypic analysis of T cell lines.

Cells were washed three times with PBS supplemented with 9% w/v BSA, 0.1% w/v sodium azide (Sigma) (PBS-BSA-Na) and resuspended at 10^6/ml in the same buffer in microtiter plate with optimal dilutions of monoclonal antibodies. After three washes in cold PBS-BSA-Na, 50 µl of 1/200 dilution of a fluoresceinated goat anti-mouse IgG (Tago, Burlingame, CA) was added and the cells incubated at 4 °C in the dark for 30 min. After three additional washes, cells were resuspended in 200 µl PBS with 1% v/v paraformaldehyde and flow microfluorometry was performed on a fluorescence-activated cell sorter (FACS) and analyzed by LYSIS II cell analysis program.

Cytotoxicity assays.

The method is generally as described in Doolan et al. (1991), Autologous PBM were stimulated with 3 µg/ml PHA in MEM supplemented with 10% v/v normal human serum for 3 days and 30 U/ml of IL-2. The PHA blasts were rested for 3 days then washed 3 times and labelled for 1 hr at 37 °C with 100 µCi of ^{51}Cr, with or without 10 µg/ml of peptide 145, 146 or M protein. After three washes, 5000 ^{51}Cr-labeled target cells were added to 96-well round bottom plate with varying number of effector cell lines at 100, 80, 40, 20, 10, 5, 2.5 and 1.25 ratios of effector-to-target (E/T) cells. Cells were incubated at 37 °C for 6 hours. After the incubation, plates were centrifuged at 100 µl of supernatants were harvested at ^{51}Cr release was measured with a gamma counter. Maximum release was measured after detergent lysis. Percent specific lysis was calculated as the following formula:

$$10x \quad \frac{[\text{mean experimental cpm}] - [\text{mean spontaneous release cpm}]}{[\text{mean maximum release cpm}] - [\text{mean spontaneous release cpm}]}$$

ELISA.

The method was as generally described in Example 1.

2. Results

The overlapping synthetic peptides (20-mers) spanning the conserved carboxylterminal region of the M protein were used to define B and T cell epitopes in Aboriginal donors comprising patients with histories of RF, RHD, other heart diseases (mainly ischaemic) and normal controls, and Caucasian donors comprising those with past histories of RF, other heart diseases (mainly ischaemic) and normal controls.

B Cell Epitope Mapping.

Sera from the different individuals were assayed by ELISA for the presence of antibodies specific for the overlapping peptides. The sera were diluted 1:40 and added to wells to which peptides were attached. The mean optical densities for sera from different groups of Aborigines and Caucasians are given in Figure 3. Population-specific differences were observed. However, a striking feature is that all groups of Aborigines (patients and controls) have antibodies that predominantly recognize peptide 145. There was a much lower response to the overlapping peptide 146, thus mapping a dominant linear B cell epitope to the aminoterminal half of peptide 145.

T Cell Epitope Mapping.

T cell epitopes were defined using the lymphoproliferation assay. Initially, the reactivity to the peptides of cord blood lymphocytes of 17 healthy caucasian neonates born from healthy mothers was tested. Since the highest stimulation index recorded was 5, this value was used as a cutoff for the proliferation studies for the adult subjects. Amongst Caucasians, individuals with past history of RF responded more frequently than individuals with no history of RF. The peptide most frequently recognized by this group was peptide 145 (25% of individuals). Surprisingly, however, T cells from 10-25% of individuals

with other heart diseases (predominantly ischaemic heart disease) also responded to many peptides.

Amongst Aborigines, T cells from patients with past histories of RF or RHD responded more frequently than T cells from normal control subjects or patients with other heart diseases. Peptides 145, 147, 152 and 157 (in normal controls) were recognized by >20% of the population studied. Within the Aboriginal group, there was no significant difference in the peptide-specific T cell responses of patients with established RHD and those with past or present histories of RF.

Because of the potential importance of peptide 145 in developing a vaccine, T cell lines specific for peptides 145 and 146, and for an acid extract of serotype 5 M protein were generated. The donor was a normal Caucasian individual with no history of heart disease. All lines responded to both peptides 145 and 146, including the M protein-specific line which responded to both the peptides and to the M protein extract at equivalent levels. This confirms that in this individual, the T cell epitope is represented by the overlapping segment of these peptides (AKKQVEKALE), and that this epitope is the dominant epitope within the protein. Surface phenotype analysis of the lines showed that most cells expressed CD3+, CD8+, and TCRα/β+, whereas most cells expressed neither CD4 nor TCRγ/δ. The T cell lines were tested for reactivity to porcine myosin and an extract of human heart, but there was minimal reactivity. Functional analysis of these lines showed that the peptide 146-specific line was cytotoxic, being able to kill autologous PHA-stimulated T cell blasts in the presence of the peptide. Although this was a repeated finding for this line, much less cytotoxicity was observed for the other lines.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations

and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 1. Overlapping peptides representing the conserved region (145-159) of M-5, aminoterminal regions of M-5, M-6 and M-24 group A streptococci (160, 161 and 162, respectively), human skeletal muscle myosin (164) and human heart myosin (165 and 166).

PEPTIDE No.	AMINO ACID SEQUENCE
145	L R R D L D A S R E A K K Q V E K A L E
146	A K K Q V E K A L E E A N S K L A A L E
147	E A N S K L A A L E K L N K E L E E S K
148	K L N K E L E E S K K L T E K E K A E L
149	K L T E K E K A E L Q A K L E A E A K A
150	Q A K L E A E A K A L K E Q L A K Q A E
151	L K E Q L A K Q A E E L A K L R A G K A
152	E L A K L R A G K A S D S Q T P D T K P
153	S D S Q T P D T K P G N K A V P G K G Q
154	G N K A V P G K G Q A P Q A G T K P N Q
155	A P Q A G T K P N Q N K A P M K E T K R
156	N K A P M K E T K R Q L P S T G E T A N
157	Q L P S T G E T A N P F F T A A A L T V
158	P F F T A A A L T V M A T A G V A A V V
159	M A T A G V A A V V K R K E E N
160	A V T R G T I N D P Q R A K E A L D Y E
161	R V F P R G T V E N P D K A R E L N K Y
162	V A T R S Q T D T L E K V Q E R A D K F
164	A L R S Q E D L K E Q L A M V E R R A N
165	M K E E F T A L K E A L E K S E A R R K
166	M E N D K Q Q L D E K L E K K E F E I S

Footnote: Peptides 145-159 overlap by 10 amino acids

SUBSTITUTE SHEET

Table 2. Amino acid sequence homology between peptides derived from M-5 protein conserved region and human skeletal muscle(164) or human heart myosin peptides(165-166)

M-5 peptide	MYSS(164)	Position	MYSB(165)	Position	MYSP(166)	Position
145	LRRDL	76-80	LRRDLD	950-955	LRRDL	188-192
146			KKQVEKALEEAN	1878-1889		
147	ELEESK	326-331	LNKELEE	1104-1110		
148	ELEESKK	326-332			ELEESKK	529-535
149	EKEKAELQAKLE	475-486	EKEKAELQ	1534-1541	EKEKAELQAKLE	678-689
150			ALKEQLAK	858-865	LEAEAKA	463-469
151	LKEQLA	608-613	LKEQLAK	859-865	LKEQLAK	220-226
152			ELAKLR	1525-1530	LAKLR	73-77
153			PGKG	211-214		
154			PGKG	211-214		
155			APMKE	850-854		
156	LTKRQL	255-260	QLPSTGET	1515-1522	ETKRQL	458-463
157			QLPSTGET	1515-1522		
158						
159			VKRKEE	1614-1619	VKRKEE	758-763
160						
161	KAREL	759-763				
162			QTDTLE	1630-1035	QTDTLE	174-179

MYSS, Human myosin heavy chain, cardiac muscle beta isoform
MYSB, Human myosin heavy chain, perinatal cardiac muscle
MYSP, Human myosin heavy chain, skeletal muscle

underline, homology sequence
blank, no homology sequence

Table 3. Lack of T cell cross-reactivity of M peptide-specific T cells with porcine heart myosin, human heart myosin peptides, and skeletal muscle myosin peptides.

Mouse	Peptide	Stimulation Index (SI) of lymph node cells to:					
		PPD	164	165	166	Myosin	Immunizing peptide
B10.D2	149	63.03	0.82	0.80	0.91	2.16	27.46
	151	6.75	0.98	1.16	1.02	1.26	6.07
B10.BR	151	5.44	1.27	1.29	1.26	1.56	2.72
	145	8.47	1.16	1.08	1.12	1.36	2.3
	146	7.88	1.78	2.23	2.05	3.57	38.95
B10.S7(R)	151	3.73	1.05	1.06	1.04	1.19	3.23

Footnote. Antigens were tested *in vitro* at various concentrations: synthetic peptides, 0.3, 3, 30 µg/well; myosin, 3, 5, 10 µg/well. The results are given only for the optimal concentrations which were 30 µg/well for M protein peptides, 3 µg/well for myosin peptides, and 10 µg/well for myosin

Table 4. T cell and antibody responses of mice after immunization with individual peptides

Mouse strain	Peptide	Stimulation index(SI)†	ELISA titer‡
B10	150	3.91	1,600
B10	152	5.06	160
B10	157	12.12	<20
B10	158	12.95	<20
B10	160	5.21	>12,800
B10.D2	146	18.55	>12,800
B10.D2	149	12.72	>12,800
B10.D2	151	11.66	>12,800
B10.D2	152	13.34	80
B10.D2	158	2.72	<20
B10.D2	159	8.69	<20
B10.D2	161	2.15	>12,800
B10.BR	145	6.27	>12,800
B10.BR	146	12.61	>12,800
B10.BR	151	16.38	>12,800
B10.BR	153	2.43	160
B10.BR	159	2.46	400
B10.BR	160	2.38	>12,800
B10.S(7R)	151	10.51	>12,800
B10.S(7R)	160	4.03	800
B10.S(7R)	162	2.99	<20

Footnote: † SI refers to lymph node cell proliferation after peptide immunization; Peptides were tested at 0.3, 3, and 30 µg/well, but the results are given for only the optimal concentration which was 30 µg/well.

‡ capture antigen for ELISA was the specific peptide;

§ Results of bactericidal assay comparing killing following incubation in test sera compared with killing following incubation in NMS. Results are mean of 3 experiments.

SUBSTITUTE SHEET

Table 5. Definition of the minimal T and B cell epitopes

Mouse	Immunizing peptide		ELISA titer and stimulation index(SI) against peptide:							
			145	146	147	148	149	150	151	152
B10.BR	145	ELISA	6400	100						
		SI	15.49	4.15						
B10.BR	146	ELISA	800	>12800	>12800					
		SI	1.06	2.49	1.27					
B10.D2	149	ELISA				>12800	>12800	200		
		SI				0.92	15.28	2.98		
B10	150	ELISA					<100	>12800	100	
		SI					0.68	3.86	0.63	
B10.D2	151	ELISA						<100	>12800	6400
		SI						1.63	26.99	12.38
B10.S7(R)	151	ELISA						100	>12800	100
		SI						1.55	5.53	2.67

Footnote. Sequences of peptides 145-152 are given in Table 1. These peptides overlap by 10 amino acids. In the T cell proliferation assays, peptides were tested at 0.3, 3, and 30 µg/well, and the results are given for only 30 µg/well which was found to be optimal.

Table 6. Binding of anti-M peptide antibodies to porcine heart myosin

Mouse	Immunizing peptide	Mouse No.	ELISA titer against porcine heart myosin
B10	150	1	<20
		2	640
		3	<20
		4	<20
	152	pool of 4	<20
	157	pool of 4	20
	158	pool of 4	<20
	160	pool of 4	<20
B10.D2	149	pool of 2	>2,560
	151	1	<20
		2	<20
		3	<20
	152	pool of 4	<20
B10.BR	145	pool of 4	160
	146	pool of 4	640
	151	1	640
		2	>2,560
		3	<20
		4	<20
	152	pool of 4	<20
	153	pool of 4	<20
	159	pool of 4	<20
B10.S(7R)	151	1	<20
		2	>2,560
		3	<20
		4	320

Table 7. Cross-reactivity of M peptide-specific antibodies with procine heart myosin

Mouse strain	Immunizing peptide	Mouse No.	Immunization schedule	ELISA O.D.(405) against:	
				Immunizing peptide	Myosin
B10.D2	149	1	Pre-immune	0.172	0.380
			Booster-1	2.000	1.044
			Booster-2	2.000	0.869
		2	Pre-immune	0.291	0.357
			Booster-1	2.000	0.401
			Booster-2	2.000	0.538
		3	Pre-immune	0.242	0.340
			Booster-1	2.000	0.464
			Booster-2	2.000	0.533
		4	Pre-immune	0.244	0.445
			Booster-1	1.708	1.085
			Booster-2	2.000	0.676
B10.S7(R)	151	1	Pre-immune	0.183	0.357
			Booster-1	2.000	1.436
			Booster-2	2.000	0.878
		2	Pre-immune	0.191	0.377
			Booster-1	1.215	0.473
			Booster-2	0.338	0.528
		3	Pre-immune	0.246	0.430
			Booster-1	0.377	0.413
			Booster-2	2.000	0.429
		4	Pre-immune	0.219	0.369
			Booster-1	2.000	0.309
			Booster-2	2.000	0.288

Footnote. Sera were tested at a 1/40 dilution. The mice depicted here are separate from the animals in Table 6.

Table 8

Bactericidal activity of antisera to peptide 145 (B10.BR mice)

Antiserum	Colony Forming Units[a]
Control[b]	143 ± 29 (n = 14 plates)
145	31, 48, 1, 6, 9, 10, 35, 51, 21, 16 ($\bar{x}$ = 23, 84% reduction)

[a] Mixture was diluted 10-fold before plating.

[b] (Normal Serum)

REFERENCES:

Ayakawa, G.Y., A.S. Bleiweis, P.J. Crowley, and M.W. Cunningham, 1988. J. Immunol. 140: 253.

Beachey, E.H., Bronze, M., Dale, J.B. Kraus, W. Poerier, T., and Sargent, S., 1988. Vaccine 6: 192.

Cunningham, M.W., and Rusell, S.M. 1983. Infect. Immun. 42: 531.

Cunningham, M.W., Hall, N.K., Krisher, K.K. and Spanier, A.M. 1986. J. Immunol. 136: 293.

Cunningham, M.W., McCormack, J.M., Talaber, L.R., Harley, J.B., Ayoub, E.M., Muneer, R.S., Chun, L.T., and Reddy, D.V. 1988. J. Immunol. 141: 2760.

Dale, J.B., Beachey, E.H. 1986. J. Exp. Med. 164: 1785.

Doolan, D.L. et al. 1991. Int. Immunol. 3: 511-516.

Fenderson, P.G. Fischetti, V.A., and Cunningham, M.W. 1989. J. Immunol. 142: 2475.

Gammon, G., Shastri, N., Gogswel, J., Wilbur, S., Sadegh-Nasseri, S., Kryzch, U. Miller, A., and Sercarz, E., 1987. Immunol. Rev. 98: 53.

Good, M.F., Branigan, J., Smith, G., and Houghten, R.A. 1990. Peptide Research 3: 110.

Good, M.F. et al. 1987. J. Immunol. 138: 306-311.

Hollingshead, S.K., Fischetti, V.A. and Scott, J.R. 1986. J. Biol. Chem. 261: 1677.

Houghten, R. A. 1985. Proc. Natl. Acad. Sci. USA 82: 5131.

Kaplan, E.L., Johnson, D.J. and Cleary, P.P. 1989. J. Infect. Dis. 159:101.

Kurabayashi, M., Tsuchimochi, H., Komura, I., Takaku, F., and Yazaki, Y. 1988. J. Clin. Invest. 82:524.

Laemmli, U.K. 1970. Nature. 277:680.

Miller, L., Gray, L., Beachey, E.H. and Kehoe, M. 1988. J. Biol. Chem. 263:5668.

Mouw, A.R., Beachey, E.H., and Burdett, V. 1988. J. Bacteriol. 170:676.

Rotta, J. and Facklam, R.R. 1980. Manual of Microbiological Diagnostic Methods for Stretococcal Infections and Their Sequelae. World Health Organisation.

Saez, L. and Leinwand, L.A. 1986. Nucleic Acid Res. 14:2961.

Tam. 1988. Proc. Natl. Acad. Sci. USA 85: 5409.

CLAIMS:

1. A synthetic peptide comprising at least one B cell epitope from the carboxyterminus of M protein of Group A β-hemolytic streptococci wherein an antibody reactive to the B cell epitope is only minimally reactive to human heart tissue.

2. A synthetic peptide according to claim 1 further comprising at least one T cell epitope from the carboxyterminus of M protein and/or from an extraneous part of, or a fusion molecule with, the synthetic peptide.

3. A synthetic peptide according to claim 1 or 2 when produced by recombinant means.

4. A synthetic peptide according to claim 1 or 2 when produced by chemically synthetic means.

5. A synthetic peptide according to claim 1 or 2 comprising an amino acid sequence substantially derived from the conserved region of the M protein or single or multiple amino acid substitutions, deletions and/or additions therein or chemical analogues thereof.

6. A synthetic peptide according to claim 5 wherein the amino acid sequence is derived from amino acid residues 337 to 492 of type 5 M protein or a single or multiple amino acid substitutions, deletions and/or additions therein or chemical analogues thereof.

7. A synthetic peptide according to claim 6 having the amino acid sequence:

L R R D L D A S R E A K K Q V E K A L E

8. A method for vaccinating a human subject against streptococcal infection comprising administering to said subject a humoral immunity developing effective amount of a peptide comprising at least one B cell epitope from the M protein of Group A β-hemolytic streptococci and at least one T cell epitope, wherein an antibody reactive with the B cell epitope is only minimally reactive with heart tissue, for a time and under conditions sufficient for said immunity to develop.

9. A method according to claim 8 wherein the T cell epitope is from the M protein carboxyterminus.

10. A method according to claim 8 wherein the T cell epitope is from an extraneous part of or fusion molecule with, the synthetic peptide.

11. A method according to claim 8 or 10 or 11 wherein the synthetic peptide is produced by recombinant means.

12. A method according to claim 8 or 9 or 10 wherein the synthetic peptide is produced by chemically synthetic means.

13. A method according to claim 8 or 9 or 10 wherein the synthetic peptide comprises an amino acid sequence substantially derived from the conserved region of the M protein or single or multiple amino acid substitutions, deletions and/or additions therein or chemical analogues thereof.

14. A method according to claim 13 wherein the amino acid sequence is derived from amino acid residues 337 to 492 of type 5 M protein or a single or multiple amino acid substitutions, deletions and/or additions therein or chemical analogues thereof.

15. A method according to claim 14 wherein the synthetic peptide comprises the amino acid sequence:

L R R D L D A S R E A K K Q V E K A L E

16. A vaccine for developing humoral immunity to M protein in a human said vaccine comprising a peptide carrying at least one B cell epitope from the M protein and at least one T cell epitope wherein an antibody reactive with said B cell epitope is only minimally reactive with heart tissue, said vaccine further comprising one or more pharmaceutically acceptable carriers and/or diluents.

17. A vaccine according to claim 16 wherein the T cell epitope is from the M protein carboxyterminus.

18. A vaccine according to claim 16 wherein the T cell epitope is from an extraneous part of, or fusion molecule with, the synthetic peptide.

19. A vaccine according to claim 16, 17 or 18 wherein the synthetic peptide is produced by recombinant means.

20. A vaccine according to claim 16, 17 or 18 wherein the synthetic peptide is produced by chemically synthetic means.

21. A vaccine according to claim 16 wherein the synthetic peptide comprises an amino acid sequence substantially derived from the conserved region of the M protein or single or multiple amino acid substitutions, deletions and/or additions therein or chemical analogues thereof.

22. A vaccine according to claim 21 wherein the synthetic peptide comprises an amino acid sequence derived from amino acid residues 337 to 492 of type 5 M protein or a single or multiple amino acid substitutions, deletions and/or additions therein or chemical analogues thereof.

23. A vaccine according to claim 22 wherein the synthetic peptide comprises the amino acid sequence:

L R R D L D A S R E A K K Q V E K A L E

24. An antibody capable of binding to a synthetic peptide comprising at least one B cell epitope from the carboxyterminus of M protein of Group A β-hemolytic streptococci, wherein said antibody is only minimally reactive to human heart tissue.

25. An antibody according to claim 24 wherein said antibody is polyclonal.

26. An antibody according to claim 24 wherein said antibody is monoclonal.

27. An antibody according to claim 24 or 25 or 26 wherein the synthetic peptide comprises the amino acid sequence:

L R R D L D A S R E A K K Q V E K A L E

28. A method for the diagnosis of streptococcal infection in a subject comprising contacting a biological sample from said subject with an antibody binding effective amount of a synthetic peptide according to any one of claims 1 to 8 for a time and under conditions sufficient for an antibody-synthetic peptide complex to form and then detecting said complex.

FIG 1

FIG 1A	FIG 1E
FIG 1B	FIG 1F
FIG 1C	FIG 1G
FIG 1D	FIG 1H

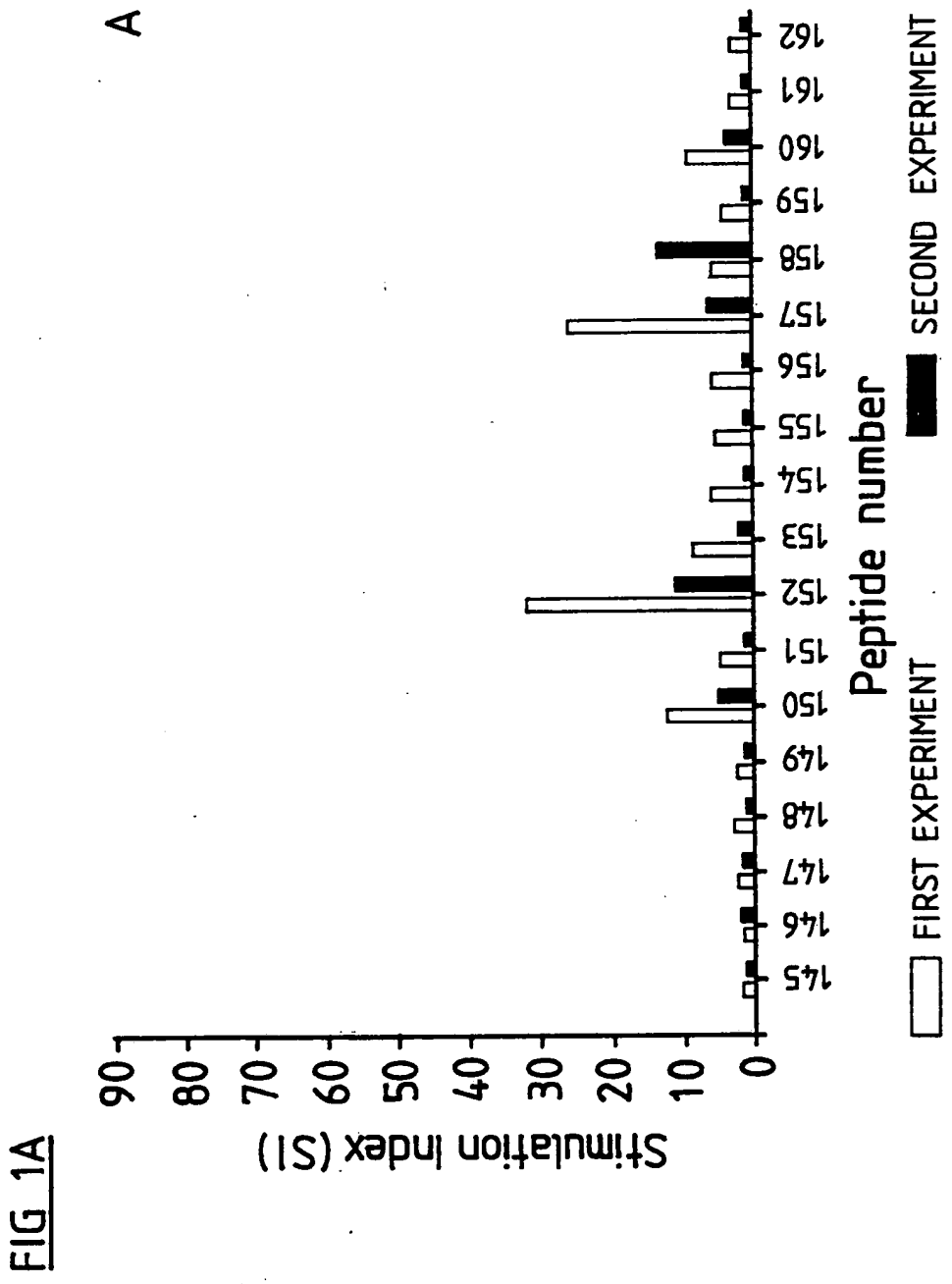

FIG 1A

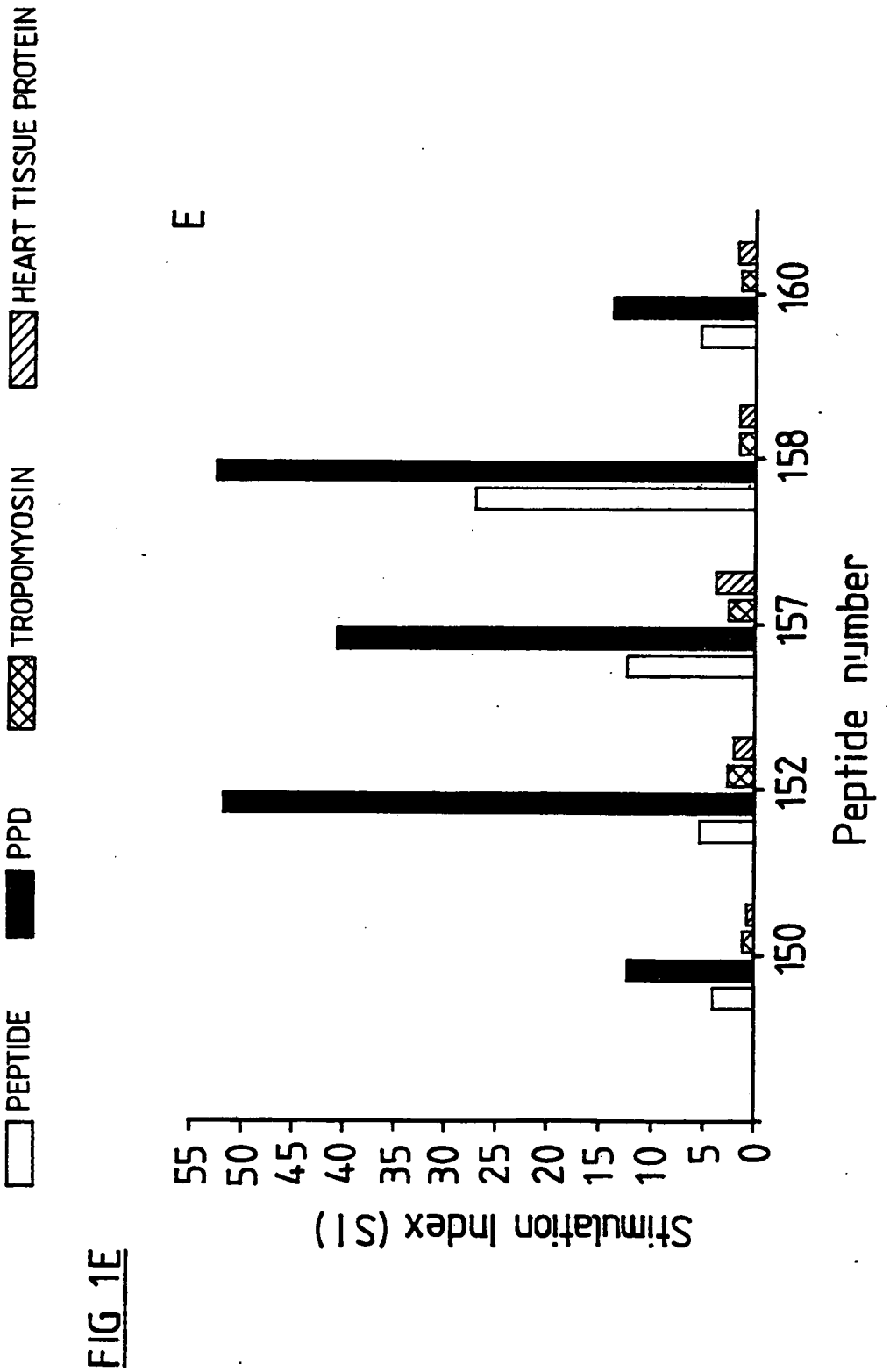
E
PEPTIDE
PPD
TROPOMYOSIN
HEART TISSUE PROTEIN
Stimulation Index (SI)
0
5
10
15
20
25
30
35
40
45
50
55
150
152
157
158
160
Peptide number

FIG 1E

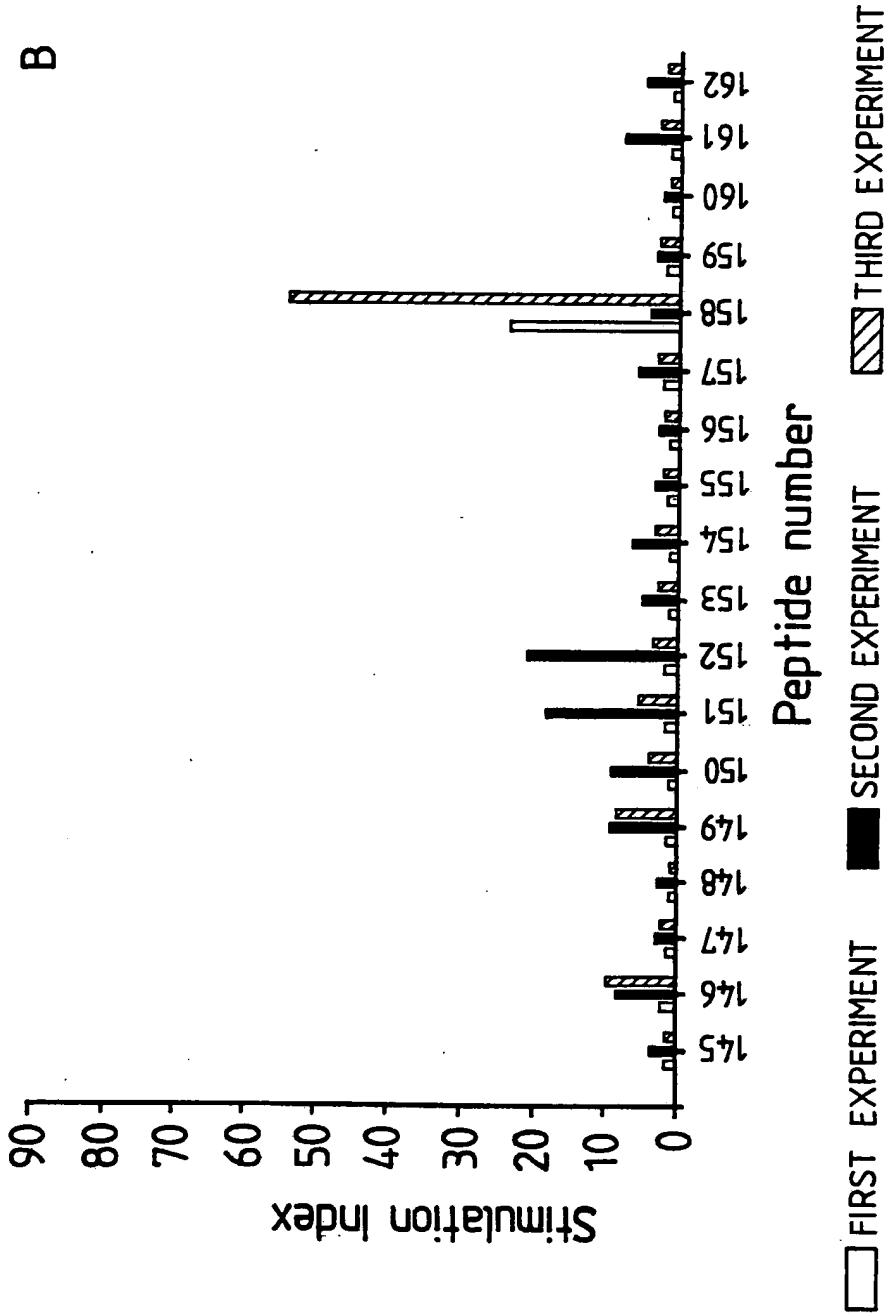
B
Stimulation Index
0
10
20
30
40
50
60
70
80
90
Peptide number
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
FIRST EXPERIMENT
SECOND EXPERIMENT
THIRD EXPERIMENT

FIG 1B

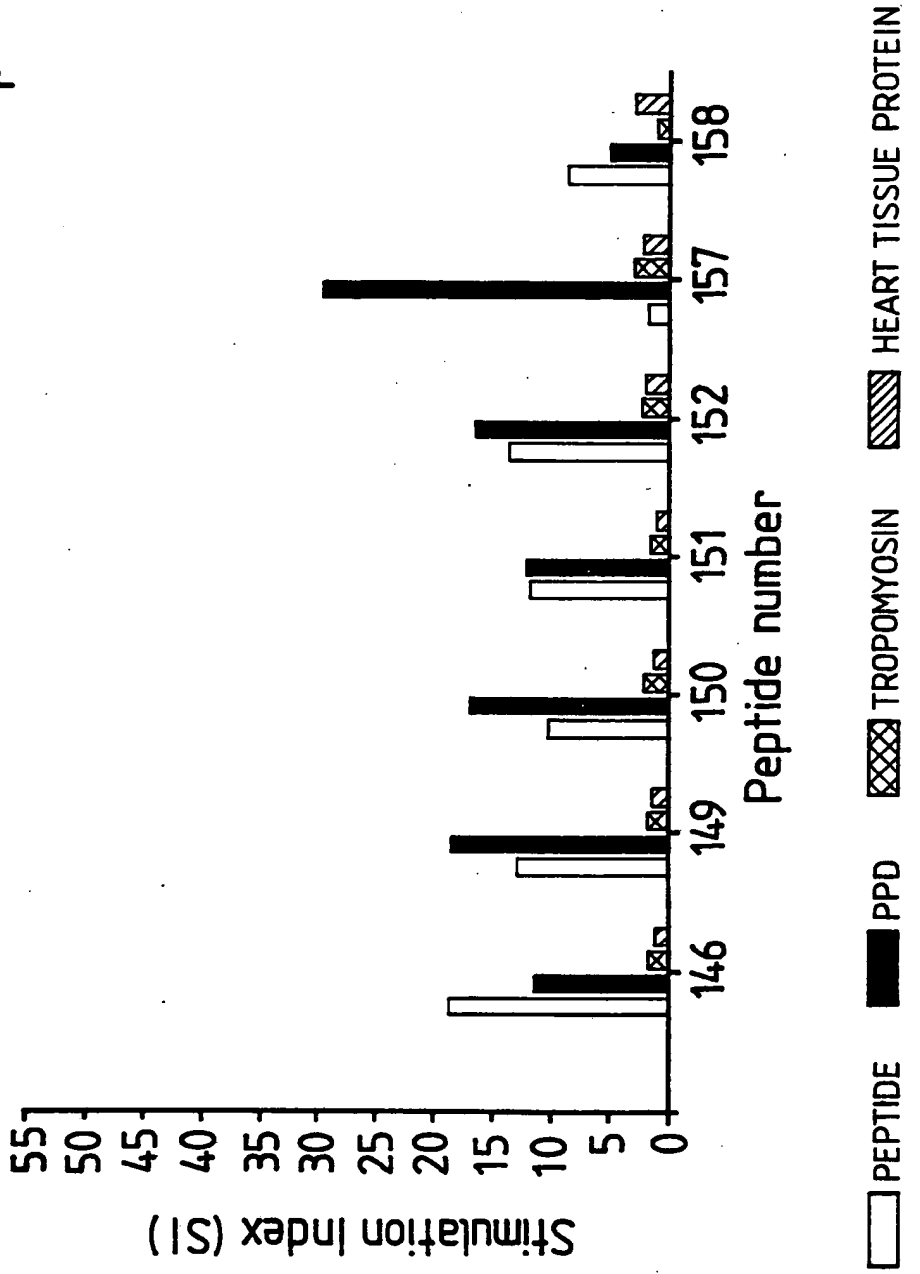
F
Stimulation Index (SI)
0
5
10
15
20
25
30
35
40
45
50
55
146
149
150
151
152
157
158
Peptide number
PEPTIDE
PPD
TROPOMYOSIN
HEART TISSUE PROTEIN

FIG 1F

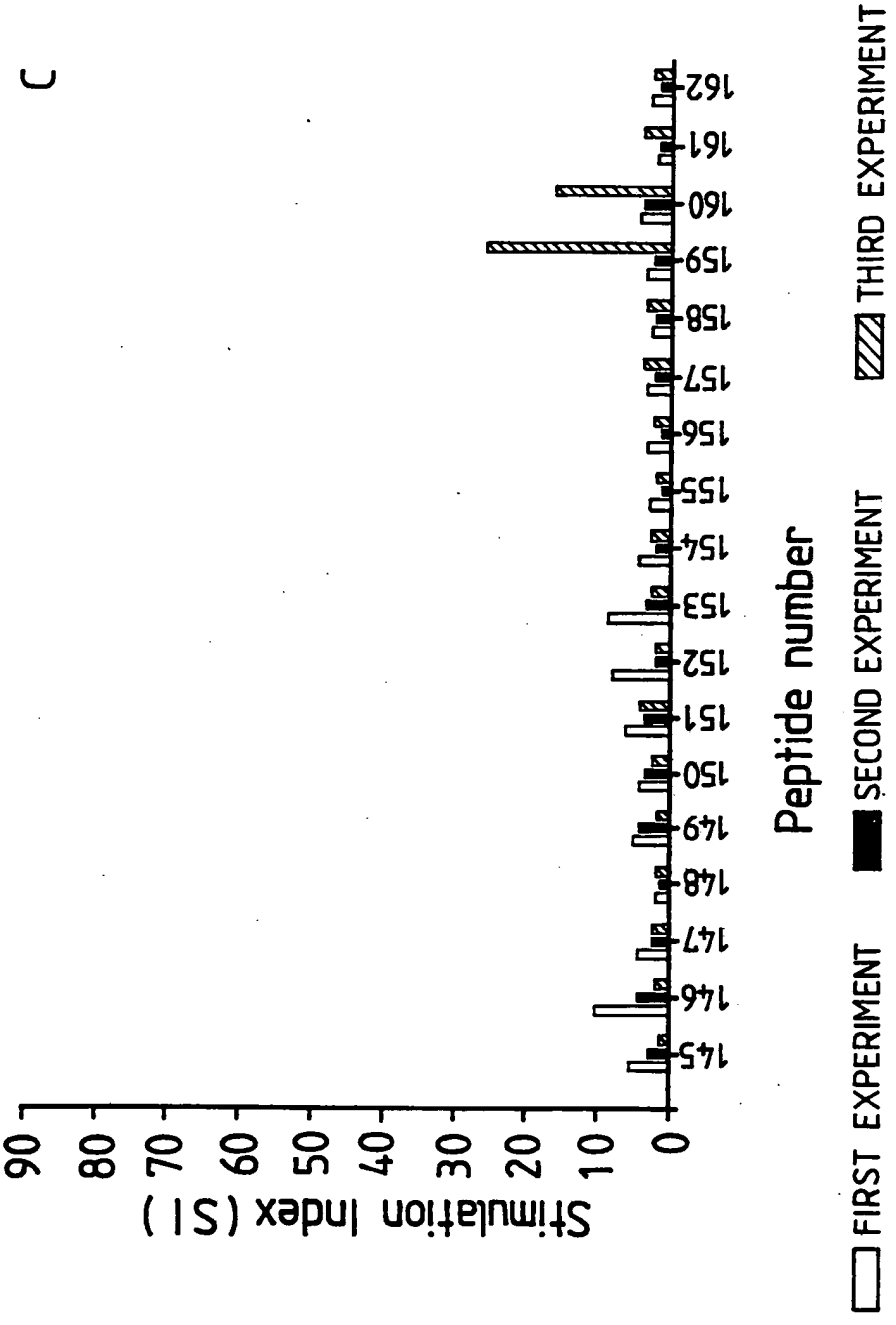
C
Stimulation Index (SI)
0
10
20
30
40
50
60
70
80
90
Peptide number
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
FIRST EXPERIMENT
SECOND EXPERIMENT
THIRD EXPERIMENT

FIG 1C

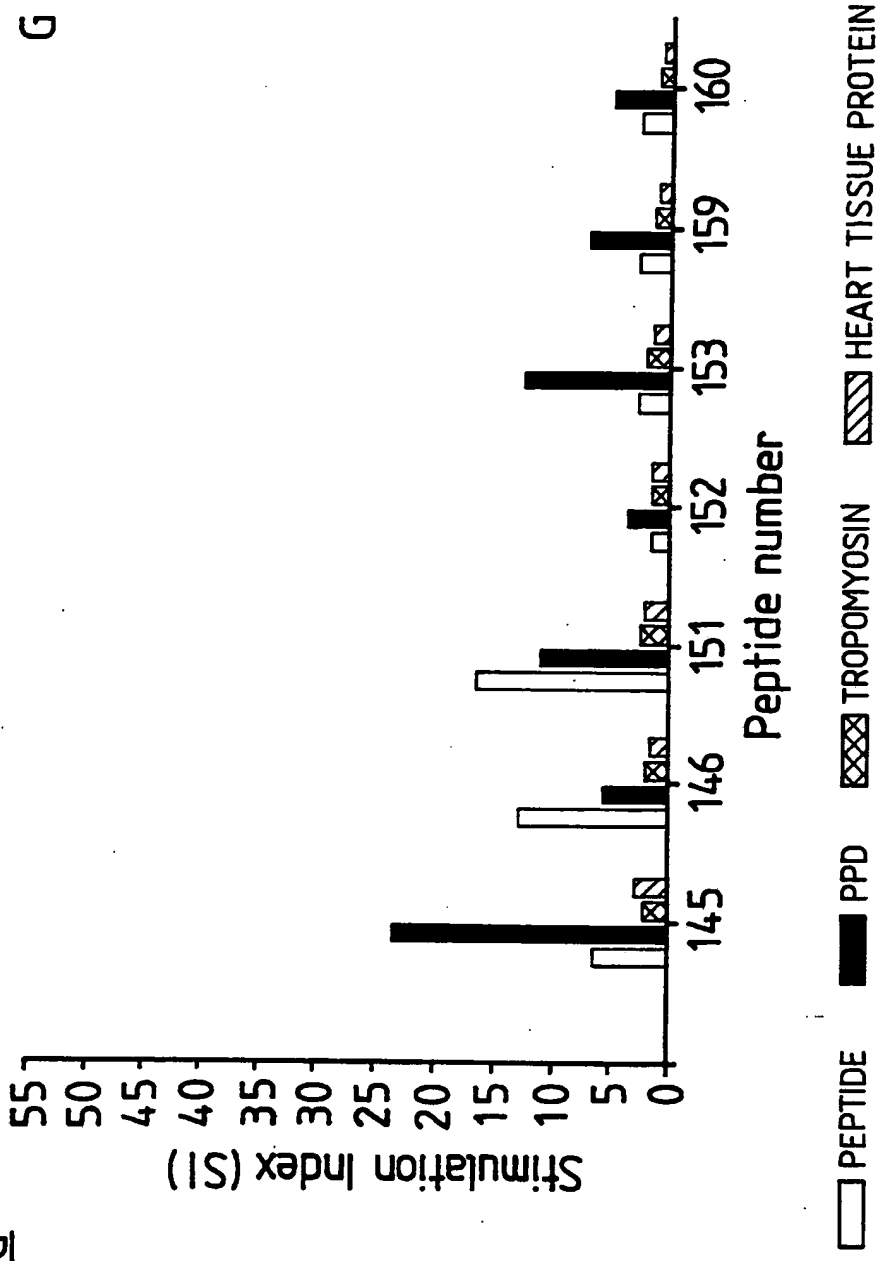
G
Stimulation Index (SI)
0
5
10
15
20
25
30
35
40
45
50
55
145
146
151
152
153
159
160
Peptide number
PEPTIDE
PPD
TROPOMYOSIN
HEART TISSUE PROTEIN

FIG 1G

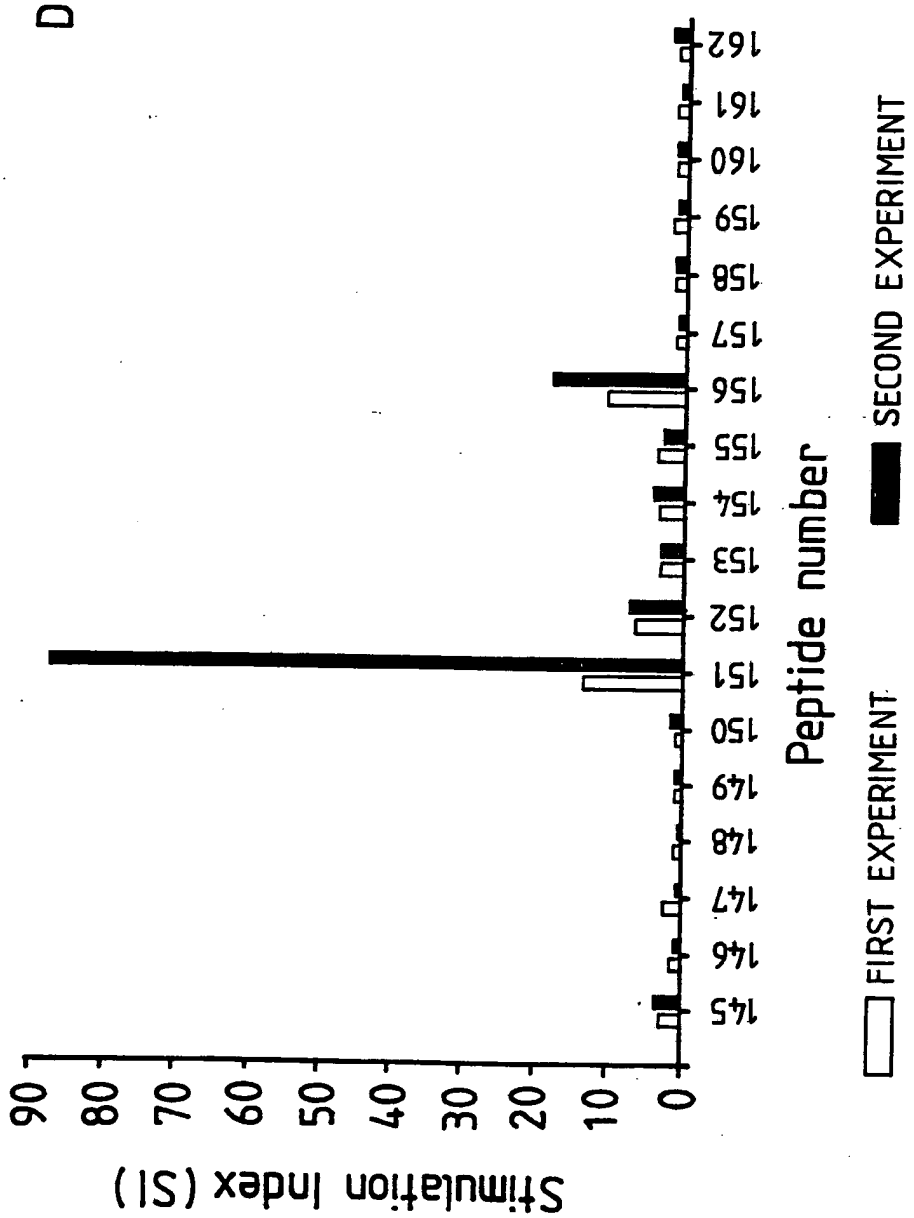
D
Stimulation Index (SI)
0
10
20
30
40
50
60
70
80
90
Peptide number
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
FIRST EXPERIMENT
SECOND EXPERIMENT

FIG 1D

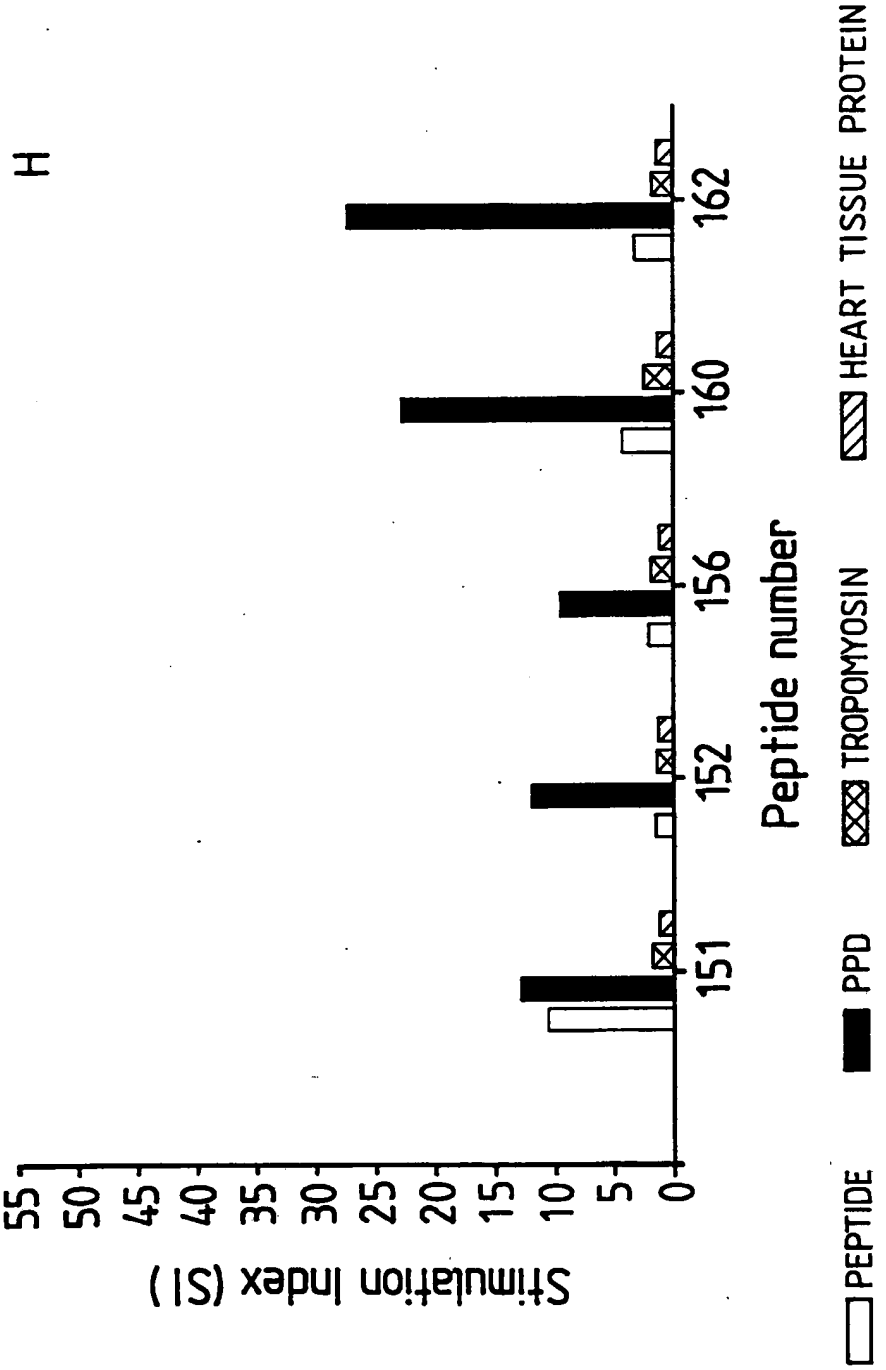
H
Stimulation Index (SI)
55
50
45
40
35
30
25
20
15
10
5
0
151
152
156
160
162
Peptide number
PEPTIDE
PPD
TROPOMYOSIN
HEART TISSUE PROTEIN

FIG 1H

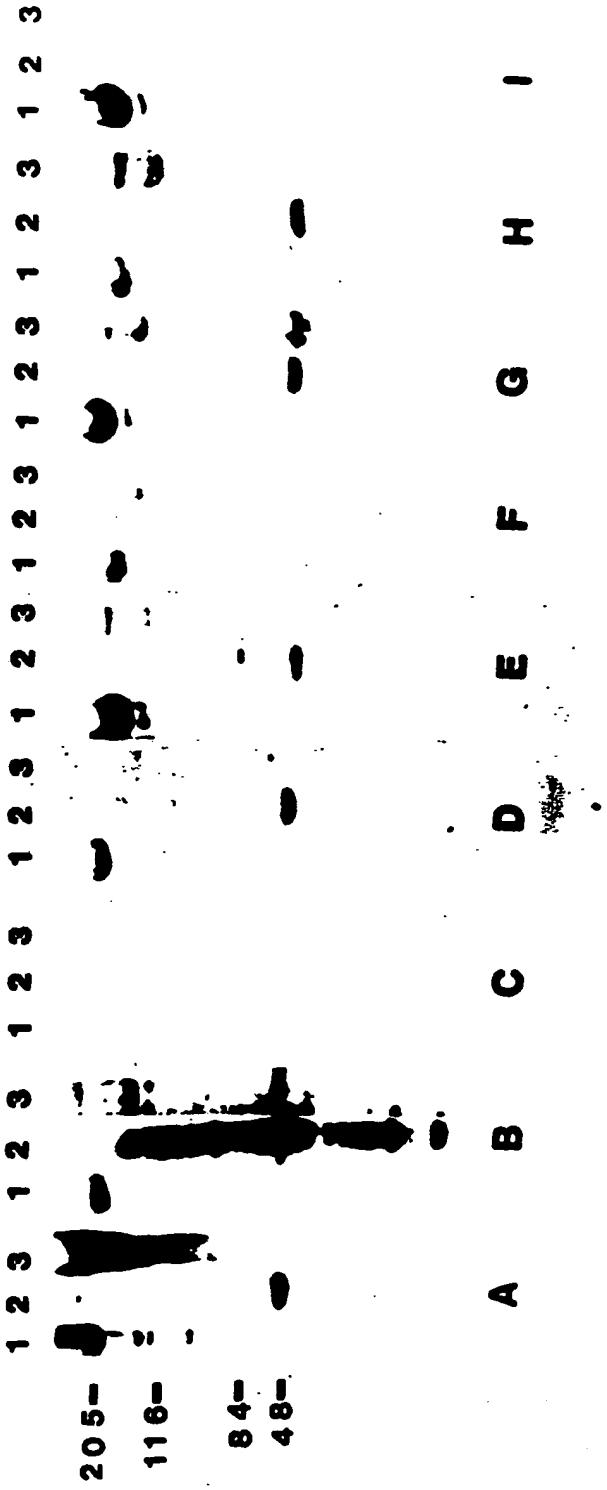

FIG 2

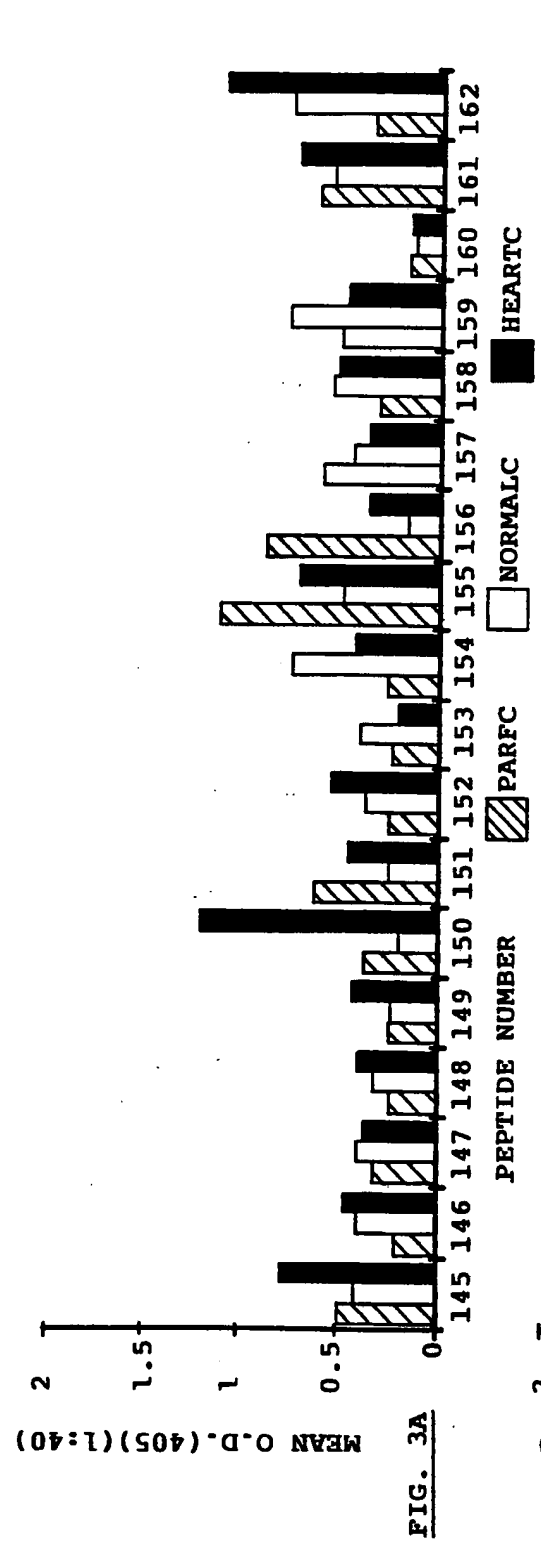

FIG. 3A

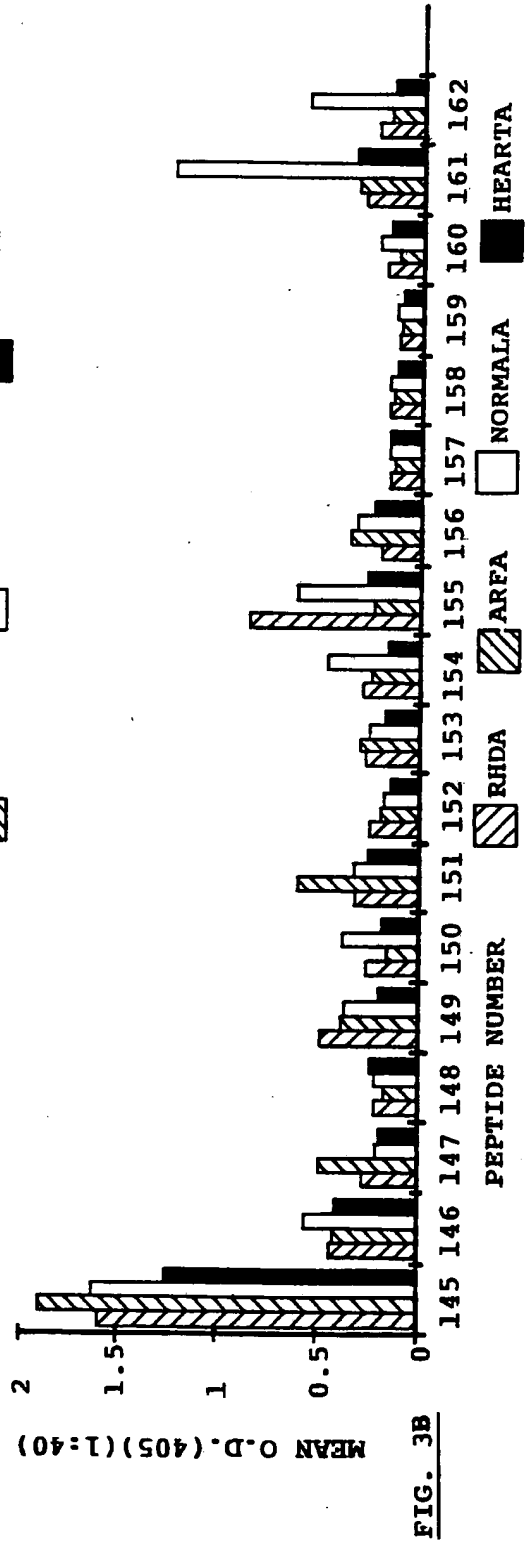

FIG. 3B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU93/00131

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[5] C07K 7/08, 7/10, 13/00; A61K 39/09, 39/40; C12P 21/08; G01N 33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC : C07K 7/08, 7/10, 13/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU : IPC as above

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT : MOPROTEIN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Infection and Immunity, Volume 59, No. 12, issued December 1991, J.H. ROBINSON et. al., "Mapping T-Cell Epitopes in Group A Streptococcal Type 5M Protein", pages 4324-4331 Abstract, Fig. 1, page 4330	1-27
A	WO,A, 90/15872 (THE ROCKEFELLER UNIVERSITY) 27 December 1990 (27.12.90) page 11-12, Fig. 1	1-27

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 May 1993 (12.05.93)	19 MAY 1993 (19.05.93)
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer T. SUMMERS Telephone No. (06) 2832291

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
A	AU,A, 34268/89 (THE ROCKEFELLER UNIVERSITY) 16 October 1989 (16.10.89) page 4, paragraph 1, Fig. 1	1-27

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member
AU 34268/89	EP 365646 WO 8909064
WO 9015872	AU 59484/90 CA 2035039 EP 437604